JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2001, p. 235–240 Vol. 39, No. 1
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.1.235–240.2001

Limited Genetic Diversity of *Brucella* spp.

BENJAMÍN GÁNDARA,[1,2] AHIDÉ LÓPEZ MERINO,[1] MARCO ANTONIO ROGEL,[3] AND ESPERANZA MARTÍNEZ-ROMERO[3]*

Escuela Nacional de Ciencias Biológicas, IPN,[1] and Centro de Investigación sobre Fijación de Nitrógeno, UNAM,[3] Cuernavaca, and Servicios de Salud de Zacatecas, S.S.A., Zacatecas,[2] Mexico

Received 30 June 2000/Returned for modification 29 August 2000/Accepted 16 October 2000

Multilocus enzyme electrophoresis (MLEE) of 99 *Brucella* isolates, including the type strains from all recognized species, revealed a very limited genetic diversity and supports the proposal of a monospecific genus. In MLEE-derived dendrograms, *Brucella abortus* and a marine *Brucella* sp. grouped into a single electrophoretic type related to *Brucella neotomae* and *Brucella ovis*. *Brucella suis* and *Brucella canis* formed another cluster linked to *Brucella melitensis* and related to *Rhizobium tropici*. The *Brucella* strains tested that were representatives of the six electrophoretic types had the same rRNA gene restriction fragment length polymorphism patterns and identical ribotypes. All 99 isolates had similar chromosome profiles as revealed by the Eckhardt procedure.

Brucellosis is a worldwide zoonosis that is especially prevalent in northern and central agricultural regions of Mexico (27). *Brucella* was once considered to be related to the genera *Bordetella* and *Alcaligenes* (18). Later on, molecular biology techniques indicated that *Brucella* had taxonomic affiliation with members of the CDC group Vd (8), and an analysis of 16S rRNA gene sequences confirmed its inclusion in the α2 subdivision of the *Proteobacteria* class (31). Since the position of the nodes in 16S rRNA gene phylogenetic trees is not without uncertainty (28), the position of *Brucella* within the α-proteobacteria has not been clearly determined. Furthermore, ribosomal genes in *Brucella* have been implicated in recombination events that promoted the division of a chromosome into two chromosomes (19).

Genetic relatedness within *Brucella* has been based on a comparison of *omp2* sequences (10) and DNA restriction maps obtained from various species (30). Molecular probes have been developed for typing *Brucella* strains (6, 14), and PCR methods are available as diagnostic techniques (22, 23). The high levels of DNA-DNA relatedness among *Brucella* species led to the conclusion that *Brucella* was a monospecific genus (43).

Recently, genes involved in symbiosis in *Sinorhizobium meliloti* (the best-studied rhizobium with regard to its symbiotic determinants) have been found to be homologous to genes implicated in the pathogenesis of *Brucella*. Rhizobia (comprising the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) form nitrogen-fixing nodules; *Brucella* spp., on the other hand, are intracellular animal pathogens. The rhizobial *bac* genes participating in bacteroid differentiation (12) are homologous to genes which play a role in *Brucella* survival in macrophages as well as in mice pathogenesis (24). A two-component regulatory system BvrR and BvrS (*Brucella* virulence) is similar to *exoS* genes of *S. meliloti*. *Brucella* mutants in BvR and BvrS have a reduced capacity to invade macrophages and do not replicate intracelullarly (39). The *S. meliloti* periplasmic protease encoding gene *degP* is more similiar to the corresponding *Brucella abortus* gene than to that of *Escherichia coli* (12).

Multilocus enzyme electrophoresis (MLEE) has been frequently used to determine the genetic relatedness in bacteria, including *E. coli* (40), *Salmonella* spp. (38), and *Vibrio cholerae* (3). This technique has proven to be valuable in the characterization of emergent epidemic clones (3). The aim of the present study was to characterize *Brucella* spp. originating from different sources by MLEE and to compare the data to data obtained for rhizobia and agrobacteria.

MATERIALS AND METHODS

Strains and cultures. *Brucella* strains (75 isolates) (Table 1) originating from Mexico and abroad were isolated from human, animal, and dairy products. Also included in this study were 4 vaccine strains, 20 type strains from all different *Brucella* species and biovars, and *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* reference strains from the Centro de Investigación sobre Fijación de Nitrógeno (UNAM) collection and *Ochrobactrum anthropi* (23) and *Agrobacterium* spp. reference strains (36). *Ochrobactrum* and *Brucella* isolates were grown in soybean Trypticase (Difco) at 37°C, in *Brucella* agar, or in PY medium. *Rhizobium* strains were grown in PY medium (3 g of yeast extract, 5 g of peptone, and 0.7 g of calcium chloride per liter). All *Brucella* single-colony isolates were tested for their Gram reaction and for agglutination with anti-*Brucella* serum. Growth rates (not shown) were estimated for *Brucella melitensis* M16 and *B. abortus* 544 in order to determine harvesting times in the logarithmic phase of growth.

MLEE. Fresh liquid (1 ml) cultures (at 0.5 turbidity, MacFarland nephelometer) were used to inoculate 40-ml portions of PY media, which were shaken for 36 to 48 h at 37°C. Cell pellets obtained by centrifugation were washed, resuspended in 300 μl of 10 mM $MgSO_4$ containing lysozyme (300 μg per ml) and incubated at room temperature for 20 min. Cell lysis was achieved by freezing and thawing at −70°C for two 15-min cycles, and the resulting extracts were maintained at −70°C.

Gel electrophoresis was carried out in starch gels, and enzymatic activities were detected as described by Selander et al. (37). The enzymes assayed were the isocitrate, malate, glucose-6-phosphate, glutamate, and pyruvate dehydrogenases, plus indophenol oxidase, hexokinase, aconitase, phosphoglucomutase, and phosphoglucose isomerase and, additionally, for the 16-enzyme assays, the xanthine, alcohol, aspartate, threonine, and leucine dehydrogenases and glucosyltransferase. The different alleles (mobility variants for each enzyme) were numbered according to mobility. Electrophoretic types (ETs) were grouped from a pairwise matrix of genetic distances using the method described by Nei and Li (32). The genetic diversity (h) for each locus was calculated as $h = 1 - \Sigma x^2[n/$

* Corresponding author. Mailing address: Centro de Investigación sobre Fijación de Nitrógeno, Ap. Postal 565-A, Av. Universidad S/N, Col. Chamilpa, Cuernavaca, Mexico. Phone: (52-73)-13-16-97. Fax: (52-73)-17-55-81. E-mail: emartine@cifn.unam.mx.

TABLE 1. Bacterial strains

Species	Strains grouped according to source or reference						
	Vaccine	Human blood and bone marrow	Dog blood	Goat milk	Cow milk and cheese	Unknown	ATCC[a]
B. melitensis bv. 1	REV-1	78, 87, 91, 113, 219, 256, 261, 376, 391, 392, 393, 400, 401, 402, 415, 450, 456, 457, 458, 461, 462, 485, LAR, P217		279, 280	371		23456 (M16)
B. melitensis bv. 2		84					23457 (63/9)
B. melitensis bv. 3		254, 255, 257, 258, 259, 306				G914, G1024, T65/40	23458 (Ether)
B. abortus bv. 1	S19, RB51	ENCB			223, 240, 264, 265, 266, 267, 268, 269, 270, 271, 272, 275, 307, 308, 309, 311, 312, 313, 314		23448 (544)
B. abortus bv. 2							23449 (86/8/59)
B. abortus bv. 3							23450 (Tulya)
B. abortus bv. 4					159, 160, 241, 242, 245		23451 (292)
B. abortus bv. 5					273, 274	49/8	23452 (B3196)
B. abortus bv. 6							23453 (870)
B. abortus bv. 7							23454 (63/75)
B. abortus bv. 9							23455 (C68)
B. suis bv. 1	S2CH	106, 387		196	129, 191, 192, 377		23444 (1330)
B. suis bv. 2							23445 (Thomsen)
B. suis bv. 3							23446 (686)
B. suis bv. 4							23447 (40/67)
B. suis bv. 5							None (513)
B. canis			ISLE, 1226				23365 (RM6/66)
B. ovis							23840 (63/290)
B. neotomae							23459 (5K33)
Marine *Brucella*						14-95	

[a] Food and Agriculture Organization/World Health Organization names and numbers are in parentheses.

$(n - 1)$], where x is the frequency of the ith allele and n is the number of ETs or isolates in the population; H is the arithmetic average of all h values.

Amplified ribosomal DNA restriction analysis (ARDRA). PCR products of 16S rRNA genes were synthesized with primers fD1 and rD1 (44) that correspond to positions 8 to 27 and positions 1524 to 1540 of the *E. coli* gene. PCR products were digested with 5 U of the restriction enzymes *Msp*I, *Hin*fI, *Hha*I, and S*au*3AI, and DNA fragments were separated in 3% agarose gels (21). The PCR product of citrate synthase of *B. melitensis* M16 DNA was obtained with the *Rhizobium tropici* primers 512 MAP (TAC-AAG-TAC-CAT-ATC-GGC-CAG-CCC-TT), corresponding to bases 858 to 873, and primer CIR97037 (CCC-ATC-ATG-CGG-AAC-GGA-TC), corresponding to bases 1218 to 1237.

DNA extraction and Southern blot hybridization. DNA was purified, blotted onto nylon filters, and hybridized to the PCR 16S rRNA gene product from *B. melitensis* M16, to the total DNA from the same strain labeled with ^{32}P by RediPrime (Amersham), or to the PCR-synthesized citrate synthase gene. DNA-DNA hybridization was performed from Southern blots, and washings were performed either at 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (high) or at 1× SSC (low) stringency.

Eckhardt gel electrophoresis. The modified procedure by Hynes and McGregor (16) involving a gentle lysis of the cell pellet with lysozyme and sodium dodecyl sulfate (incorporated into the agarose gel [agarose Sigma Type 1:Low EEO, catalog number A-6013]) was used with early-log-phase bacteria grown in PY medium. Horizontal gels were run at 80 V for 10 h at room temperature. Plasmid sizes were estimated using *S. meliloti* megaplasmids (1.4 and 1.7 Mb [2]) as references. The miniscreening procedure (4) was also used in order to visualize small plasmids.

RESULTS

Most human isolates from Mexico corresponded to *B. melitensis* bv. 1, and a majority of dairy product isolates corresponded to *B. abortus* bv. 1 based on the traditional classification methods (27). We did not isolate *Brucella ovis* and *Brucella neotomae*. The H value among the four *Brucella* ETs obtained with 10 enzymes was 0.16, and the H value calculated for all isolates was 0.04. Representatives from each ET and some *R. tropici* and *Ochrobactrum* strains were analyzed with an additional six enzymes in order to reveal further diversity (Table 2). This resulted in two of the *Brucella* spp. ETs being split into two related ETs, while the other ETs remained unaltered. Each *Brucella* species was distinguishable by its ET with 16 enzymes (Table 2; see Fig. 2). The total number of ETs obtained with *Brucella* isolates was six, and the H value among the six ETs was 0.32. If we consider that six ETs represent all of the 99 strains tested, then the strain/ET ratio would be 16.5, a value higher than that encountered (ca. 1) from single-species *Rhizobium* populations. The high strain/ET ratio encountered in *Brucella* spp. is an indicator of a limited genetic diversity that is also revealed by the low number of polymorphic enzymes detected (9 of 16).

The MLEE-derived dendrogram obtained with *Brucella* spp. and rhizobia confirms their close relationship (Fig. 1 and 2). With the 16-enzyme analysis, two subclusters may be distinguished for *Brucella* isolates, one with the marine *Brucella*, *B. abortus*, *B. neotomae*, and *B. ovis*, and the other with *B. canis*, *B. suis*, and *B. melitensis. R. tropici* strains grouped with the second subcluster. *O. anthropi* and *Agrobacterium* spp. were related to *Brucella* at a genetic distance of 0.9 (Fig. 1).

TABLE 2. Allele profiles of the 16 metabolic enzymes tested[a]

Strain	Allele profile of:															
	IDH	MDH	G6P	GDH	PDH	IPO	HEX	ACO	PGM	PGI	XDH	ADH	ASD	THD	LED	GTF
O. anthropi 95/5	8	5	5	8	8	4	8	8	8	8	8	8	8	8	8	8
B. suis 1330	7	3	3	7	7	6	7	7	7	7	5	7	7	7	7	5
B. suis 4 40/67	7	3	3	7	7	6	7	7	7	7	5	7	7	7	7	5
B. canis RM6/66	7	3	3	7	7	6	7	7	7	7	7	7	7	7	7	5
B. canis 1226	7	3	3	7	7	6	7	7	7	7	7	7	7	7	7	5
R. tropici CIAT 899	7	7	2	7	7	6	7	7	7	7	8	7	7	7	7	5
R. tropici CFN 299	7	5	2	7	7	4	7	7	7	7	7	7	7	7	7	5
B. melitensis M16	7	5	5	7	7	7	7	7	7	7	7	7	7	5	7	5
B. melitensis 84	7	5	5	7	7	7	7	7	7	7	7	7	7	5	7	5
B. abortus 3 Tulya	7	5	5	7	7	7	7	7	7	7	5	5	5	5	5	5
B. abortus 544	7	5	5	7	7	7	7	7	7	7	5	5	5	5	5	5
Marine *Brucella* 14/95	7	5	5	7	7	7	7	7	7	7	5	5	5	5	5	5
B. neotomae 5K33	7	5	5	7	7	6	7	7	7	7	5	5	5	5	5	5
B. ovis 63/290	7	5	5	7	7	9	9	7	7	7	5	8	5	5	5	5

[a] Enzyme abbreviations: IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; GDH, NADP-dependent glutamate dehydrogenase; PDH, pyruvate dehydrogenase; IPO, indophenol oxidase; HEX, hexokinase; ACO, aconitase; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; XDH, xanthine dehydrogenase; ADH, alcohol dehydrogenase; ASD, aspartate dehydrogenase; THD, threonine dehydrogenase; LED, leucine dehydrogenase; GTF, glucosyltransferase.

A single pattern with three common bands was observed when *Eco*RI-DNA digestion fragments of several *Brucella* species (*B. melitensis* M16 and 84; *B. abortus* bv. 1, 544, and bv. 3 Tulya; *B. neotomae* 5K33; *B. suis* bv. 1, 1330, bv. 4, 40/67; the marine *Brucella* sp. strain 14/95; *B. canis* 1226) were hybridized in Southern blottings using the 16S rRNA gene PCR product of *B. melitensis* M16 as a probe. Three *rrn* loci have been found in *Brucella* spp. (30). ARDRA analysis revealed that the *Brucella* strains listed above shared a common rRNA gene pattern (data not shown).

DNA-DNA homology values indicated that *Ochrobactrum* and *Brucella* spp. were more homologous (ca. 30%) than *Bru-*

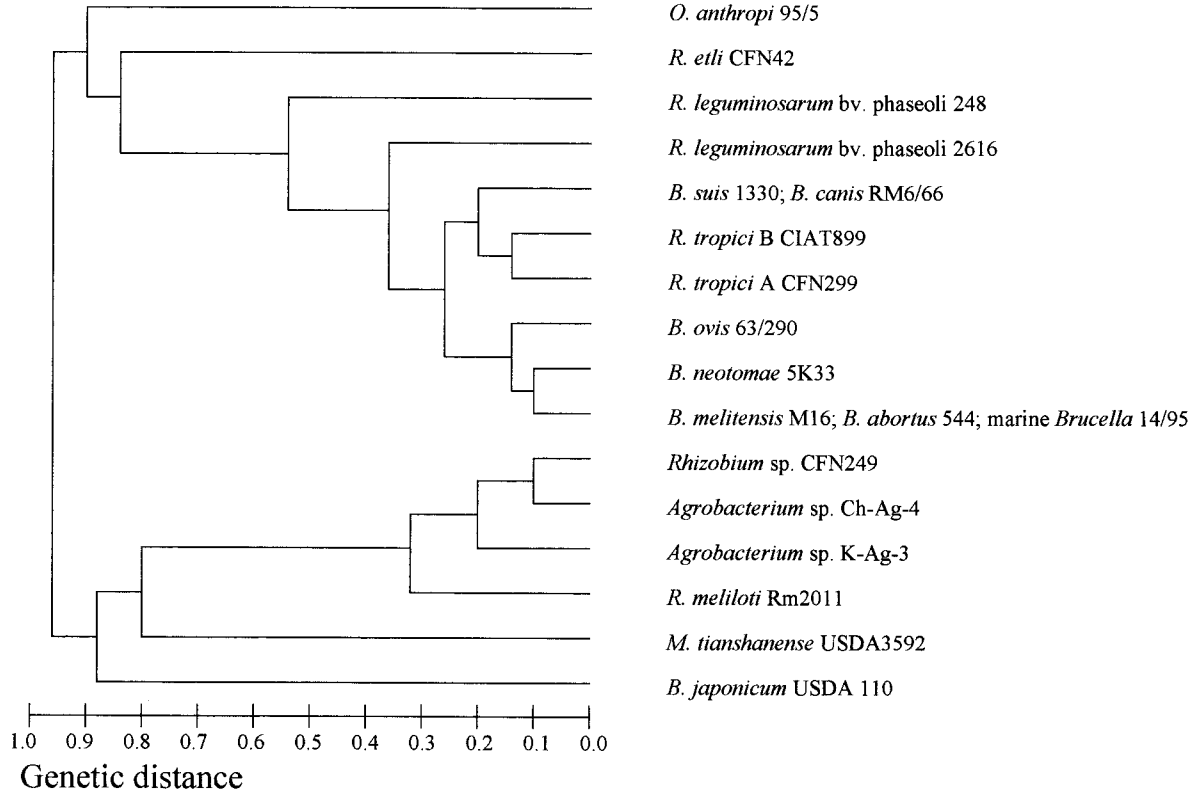

FIG. 1. Dendrogram derived from MLEE with 10 enzymes analyzed: isocitrate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, NADP-dependent glutamate dehydrogenase, pyruvate dehydrogenase, indophenol oxidase, hexokinase, aconitase, phosphoglucomutase, and phosphoglucose isomerase.

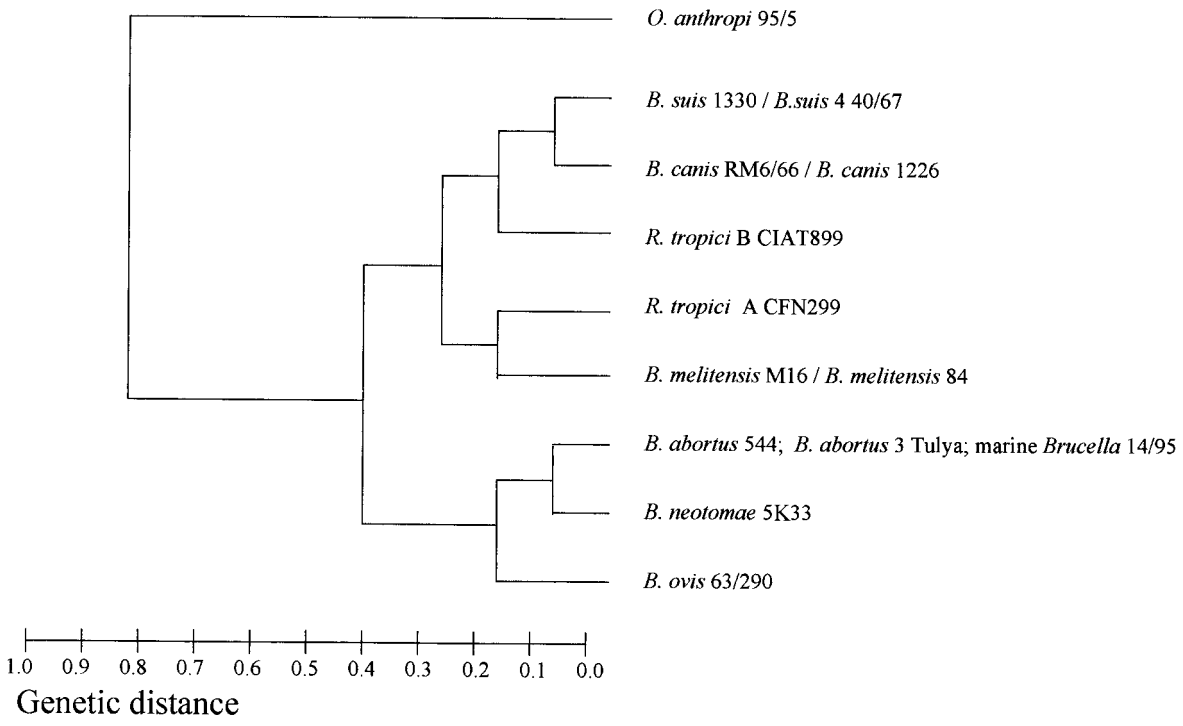

FIG. 2. Dendrogram derived from MLEE with 16 enzymes analyzed (see Materials and Methods).

cella spp. and rhizobia (ca. 10%). At high stringency, a slightly higher percentage of hybridization is obtained with *R. tropici* than with *S. meliloti*, but this may not be significant.

Interestingly, *Brucella* chromosomes were easily visualized with the Eckhardt procedure that we normally use to reveal plasmids and megaplasmids in rhizobia. Most of the strains had the same pattern corresponding to chromosomes of 2.05 and 1.15 Mb. No large or small plasmids were encountered in any of the 99 isolates tested. Two chromosomes of 1.35 and 1.85 Mb were observed with *B. suis* bv. 2 and bv. 4 in agreement with the data of Jumas-Bilak (19). The only discrepancy with the reported results was obtained with *B. suis* biovar 3 which contained smaller chromosomes (2.1 and 1.15 Mb) than that (3.2 Mb) observed by Jumas-Bilak (19). In order to resolve this discrepancy, we analyzed chromosomes from at least two additional *B. suis* bv. 3 strains from different sources, and the data confirmed our previous findings (Fig. 3). Both chromosomes from each *Brucella* strain hybridized to the 16S rRNA gene probe, while only the larger one hybridized to the citrate synthase gene (not shown). The megaplasmid of *R. tropici*, which is similar in size to the smaller chromosome of *B. suis* bv. 2 and 4, did not hybridize to the homologous 16S rRNA DNA gene probe as was reported previously (11).

DISCUSSION

MLEE analysis is a useful standard method for evaluating bacterial genetic diversity. In general, a different species is recognized if a genetic distance larger than 0.5 is observed. The limited genetic variation exhibited by the *Brucella* isolates is

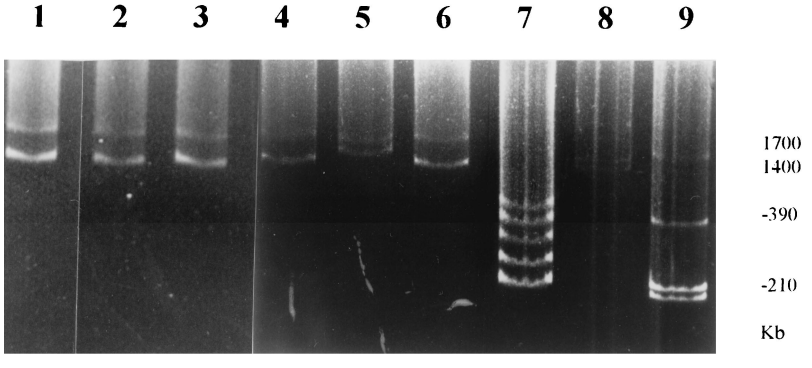

FIG. 3. Plasmid megaplasmids and chromosomes as visualized by the modified Eckhardt procedure. Lanes: 1, *B. abortus* bv. 1; 2, *B. melitensis* bv. 1; 3, *B. abortus* bv. 4; 4, *B. suis* bv. 3; 5, *B. suis* bv. 4; 6, *B. suis* bv. 5; 7, *R. etli* CFN42; 8, *R. meliloti* 1021; 9, *R. tropici* type A reference strain CFN299.

congruent with a monospecific genus (43). The fact that only a few clones (ETs) were obtained that are conserved through space and time probably reflects a recent origin of the genus. The *H* value for all *Brucella* species was 0.04 or 0.32 (depending if the analysis is on an isolate or ET basis), which is smaller than those normally estimated for a single *Rhizobium* species (ca. 0.5) (29). A large diversity has been encountered in rhizobia, suggesting that they represent very old lineages. Pathogens, especially those occurring intracellularly, have a narrow diversity which may reflect their habitat constraints (reviewed in reference 29). Thus, limited genetic diversity has also been obtained with *Yersinia ruckeri* and *Salmonella enterica* serovar Paratyphi B.

A close relationship of *B. suis* and *B. canis* was recognized based on phenotypic characteristics (1), and these organisms were not distinguishable by their physical maps (30). From our data, *B. suis* is very similar to *B. canis* and is only distinguishable by 1 enzyme, xanthine dehydrogenase, of 16. In general, our dendrograms obtained by MLEE are in good agreement with the one constructed on the basis of the sequence of *omp2* (10), and both methods are in general agreement with the tree derived from genome restriction maps (30). Originally, one isolate from a marine animal was considered related to *B. abortus* or *B. melitensis* (9). We could not distinguish the marine *Brucella* sp. from *B. abortus* by MLEE; however, we included only a single isolate from marine mammals. An extensive analysis of *Brucella* spp. in marine mammals showed that they possessed DNA fingerprints that differentiated them from other described *Brucella* species (5). The two *B. suis* bv. 5 strains tested had ETs corresponding to *B. melitensis* (determined with the 16-enzyme analysis) and not to the *B. suis* ET. The question regarding whether *B. suis* bv. 5 strains are bona fide *B. suis* was raised earlier based on metabolic profiles and susceptibility to phages (17).

It is possible that genetic variation may be underestimated by MLEE because different alleles may have identical mobilities (33). Variation within an ET may be revealed by DNA-based fingerprinting methods. *Salmonella enterica* serovar Typhi was found to have a worldwide limited genetic diversity and a clonal population structure as revealed with MLEE (38). Variation within serovar Typhi clones was shown with ribosomal fingerprinting. These results may be explained if ribosomal gene rearrangements (26) and recombination occurred faster than detectable changes in isoenzymes. Thus, MLEE would allow for the detection of older genetic relationships, as we suppose is the case with *R. tropici* and *Brucella*. *Mycobacterium tuberculosis*, another intracellular human and animal pathogen, which has been found to be genetically very homogeneous, is considered to have evolved relatively recently from a soil bacterium (7, 41). Our hypothesis is that both *Brucella* and *R. tropici* have a common ancestor and have conserved the type of inherited alloenzymes. We further suppose that these enzymes were adapted to an acid intracellular environment. *R. tropici* has been described as highly tolerant to acidity in comparison to many other *Rhizobium* species, including *S. meliloti* (13), whereas *Brucella* spp. must survive low gastric pH, and an adaptive acid tolerance response has been described (20). Tolerance to acidity allows the survival of other bacteria in cheese (25), and *Brucella* is normally encountered in fermented dairy products. Our suggestion of a common origin of *Brucella* and *Rhizobium* spp. certainly agrees with the proposal that a larger chromosome (as in *Rhizobium*) gave rise to the two smaller chromosomes found in *Brucella* (19).

The DNA-DNA hybridization results showed that *Brucella* was more homologous to *Ochrobactrum* (30% DNA-DNA hybridization) than to *R. tropici* (12%). It is worth noting that the percentage of total DNA-DNA homology among *Brucella* spp. and *S. meliloti* or *R. tropici* (ca. 11%) is lower than the percentage of nucleotide identity encountered when different homologous genes are compared. For example, *Brucella* and *S. meliloti degP* gene sequences are 55.5% identical; a fragment of 400 bp of *pckA* gene (for phosphoenolpyruvate carboxykinase) is 77% identical among *Sinorhizobium* sp. strain NGR234 and *B. abortus* (39, 35), and the citrate synthase gene of *Brucella* is about 80% identical to the corresponding gene in *R. tropici* (our unpublished results and reference 15). A fragment of phospholipid *N*-methyltranferase (*pmtA*) genes of *B. ovis* and *S. meliloti* are 61% identical (O. Geiger, personal communication). This may mean that some parts of the *Brucella* genome are shared with rhizobia, while others may have been acquired from other sources. The MLEE relationships observed between *Brucella* and rhizobia may be explained if the common genome encodes the metabolic enzymes we have analyzed. In contrast, similarities in genes coding for a secretion system of *B. suis* and *Bordetella pertussis* have been reported (34). It is intriguing that in spite of the fact that *Ochrobactrum* isolates, especially *O. intermedia*, are clearly related to *Brucella* (42), we did not detect a high degree of similarity among *Brucella* and *Ochrobactrum* isolates by MLEE. *Ochrobactrum* has been shown to be highly diverse. This diversity may be the result of extensive interstrain recombination with randomization of enzymatic alleles. Notably, *R. tropici* type A and type B share only 36% DNA-DNA homology, yet they are considered to constitute a single species. Finally, the easy and clear detection of the chromosomes of *Brucella* spp. in Eckhardt gels may be useful in *Brucella* research to determine whether a gene is present on a specific replicon (as we have shown with the citrate synthase gene) and for further characterization or even identification of new isolates.

ACKNOWLEDGMENTS

We thank J. Martínez Romero for technical support, L. Barran and M. Dunn for critically reviewing the manuscript, and A. MacMillan for providing the marine *Brucella* and *O. anthropi* strains.

B.G.J. was supported by a graduate student scholarship from CONACyT and PIFI-IPN, and A.L.M. was supported by a research scholarship from COFAA-IPN. This work was also supported by grants from CONACyT (27594B) and CGPI-IPN (980367).

REFERENCES

1. **Allardet-Servent, A., G. Bourg, M. Ramuz, M. Pages, M. Bellis, and G. Roizes.** 1988. DNA polymorphism in strains of the genus *Brucella*. J. Bacteriol. **170:**4603–4607.
2. **Barloy-Hubler, F., D. Capela, M. J. Barnett, S. Kalman, N. A. Federspiel, S. R. Long, and F. Galibert.** 2000. High-resolution physical map of the *Sinorhizobium meliloti* 1021 pSyma megaplasmid. J. Bacteriol. **182:**1185–1189.
3. **Beltran, P., G. Delgado, A. Navarro, F. Trujillo, R. K. Selander, and A. Cravioto.** 1999. Genetic diversity and population structure of *Vibrio cholerae*. J. Clin. Microbiol. **37:**581–590.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Bricker, B. J., D. R. Ewalt, A. P. MacMillan, G. Foster, and S. Brew.** 2000. Molecular characterization of *Brucella* strains isolated from marine mammals. J. Clin. Microbiol. **38:**1258–1262.

6. **Bricker, B. J., and S. M. Halling.** 1994. Differentiation of *Brucella abortus* bv1, 2 and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv.1 by PCR. J. Clin. Microbiol. **32:**2660–2666.
7. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
8. **De Ley, J., W. Mannheim, P. Segers, A. Lievens, M. Denjin, M. Vanhoucke, and M. Gillis.** 1987. Ribosomal ribonucleic acid cistron similarities and taxonomic neighborhood of *Brucella* and CDC group Vd. Int. J. Syst. Bacteriol. **37:**35–42.
9. **Ewalt, D. R., J. B. Payeur, B. M. Martin, D. R. Cummins, and W. G. Miller.** 1994. Characteristics of a *Brucella* species from a bottlenose dolphin (*Tursiops truncatus*). J. Vet. Diagn. Investig. **6:**448–452.
10. **Ficht, T. A., H. S. Husseinen, J. Derr, and S. W. Bearden.** 1996. Species-specific sequences at the *omp2* locus of *Brucella* type strains. Int. J. Syst. Bacteriol. **46:**329–331.
11. **Geniaux, E., M. Flores, R. Palacios, and E. Martínez.** 1995. Presence of megaplasmids in *Rhizobium tropici* and further evidence of differences between the two *R. tropici* subtypes. Int. J. Syst. Bacteriol. **45:**392–394.
12. **Glazebrook, J., A. Ichige, and G. C. Walker.** 1996. Genetic analysis of *Rhizobium meliloti bacA-phoA* fusion results in identification of *degP*: two loci required for symbiosis are closely linked to *degP*. J. Bacteriol. **178:**745–752.
13. **Graham, P. H., K. J. Draeger, M. L. Ferrey, M. J. Conroy, B. E. Hammer, E. Martínez, S. R. Aarons, and C. Quinto.** 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR 1899. Can. J. Microbiol. **40:**198–207.
14. **Grimont, F., J. M. Verger, P. Cornelis, J. Limet, M. Lefèvre, M. Grayon, B. Régnault, J. Van Broeck, and P. A. D. Grimont.** 1992. Molecular typing of *Brucella* with cloned DNA probes. Res. Microbiol. **143:**55–65.
15. **Hernández-Lucas, I., M. A. Pardo, L. Segovia, J. Miranda, and E. Martínez-Romero.** 1995. *Rhizobium tropici* chromosomal citrate synthase gene. Appl. Environ. Microbiol. **61:**3992–3997.
16. **Hynes, M. F., and N. F. McGregor.** 1990. Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. **4:**567–574.
17. **Jahans, K. L., G. Foster, and E. S. Broughton.** 1997. The characteristics of *Brucella* strains isolated from marine mammals. Vet. Microbiol. **57:**373–382.
18. **Johnson, R., and P. H. A. Sneath.** 1973. Taxonomy of *Bordetella* and related organisms of the families *Achromobacteriaceae*, *Brucellaceae*, and *Neisseriaceae*. Int. J. Syst. Bacteriol. **23:**381–404.
19. **Jumas-Bilak, E., S. Michaux-Charachon, G. Bourg, D. O'Callaghan, and M. Ramuz.** 1998. Differences in chromosome number and genome rearrangements in the genus *Brucella*. Mol. Microbiol. **27:**99–106.
20. **Kulakov, Y. K., P. G. Guigue-Talet, M. R. Ramuz, and D. O'Callaghan,** 1997. Response of *Brucella suis* 1330 and *B. canis* RM6/66 to growth at acid pH and induction of an adaptive acid tolerant response. Res. Microbiol. **148:** 145–151.
21. **Laguerre, G., M.-R. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:**56–63.
22. **Leal-Klevezas, D. S., A. López-Merino, and J. P. Martínez-Soriano.** 1995. Molecular detection of *Brucella* spp.: rapid identification of *B. abortus* biovar I using PCR. Arch. Med. Res. **26:**263–267.
23. **Leal-Klevezas, D. S., I. O. Martínez-Vázquez, A. López Merino, and J. P. Martinez-Soriano.** 1995. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. J. Clin. Microbiol. **33:**3087–3090.
24. **LeVier, K., R. W. Phillips, V. K. Grippe, R. M. Roop II, and G. C. Walker.** 2000. Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. Science **287:**2492–2493.
25. **Leyer, G. J., and E. A. Johnson.** 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. **58:**2075–2080.
26. **Liu, S.-L., and K. E. Sanderson.** 1996. Highly plastic chromosomal organization in *Salmonella typhi*. Proc. Natl. Acad. Sci. USA **93:**10303–10308.
27. **López-Merino, A.** 1991. Brucelosis humana, p. 4–17. *In* A. López-Merino (ed.), Brucelosis: avances y perspectivas, México. Publicación técnica del INDRE-SSA no. 6. Secretaría de Salud, Mexico City, Mexico.
28. **Ludwig, W., R. Amann, E. Martínez-Romero, W. Schönhuber, S. Bauer, A. Neef, and K.-H. Schleifer.** 1998. rRNA-based identification and detection systems for rhizobia and other bacteria. Plant Soil **204:**1–19.
29. **Martínez-Romero, E., and J. Caballero-Mellado.** 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. **15:**113–140.
30. **Michaux-Charachon, S., G. Bourg, E. Jumas-Bilak, P. Guigue-Talet, A. Allardet-Servent, D. O'Callaghan, and M. Ramuz.** 1997. Genome structure and phylogeny in the genus *Brucella*. J. Bacteriol. **179:**3244–3249.
31. **Moreno, E., E. Stackebrandt, M. Dorsch, J. Wolters, M. Busch, and H. Mayer.** 1990. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the α-2 subdivision of the class *Proteobacteria*. J. Bacteriol. **172:**3569–3576.
32. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76:**5269–5273.
33. **Nelson, K., T. S. Whittam, and R. K. Selander.** 1991. Nucleotide polymorphism and evolution in the glyceraldehyde-3-phosphate dehydrogenase genes (*gapA*) in natural populations of *Salmonella* and *Escherichia coli*. Proc. Natl. Acad. Sci. USA **88:**6667–6671.
34. **O'Callaghan, D., C. Cazevielle, A. Allardet-Servent, M. L. Boschirolli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essencial for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
35. **Osteras, M., T. M. Finan, and J. Stanley.** 1991. Site-directed mutagenesis and DNA sequence of *pckA* of *Rhizobium* NGR234, encoding phosphoenolpyruvate carboxykinase: gluconeogenesis and host-dependent symbiotic phenotype. Mol. Gen. Genet. **230:**257–269.
36. **Sawada, H., and H. Ieki.** 1992. Phenotypic characteristics of the genus *Agrobacterium*. Ann. Phytopathol. Soc. Japan **58:**37–45.
37. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873–884.
38. **Selander, R. K., P. Beltran, N. H. Smith, R. Helmuth, F. A. Rubin, D. J. Kopecko, K. Ferris, B. D. Tall, A. Cravioto, and J. M. Musser.** 1990. Evolutionary genetic relationships of clones of *Salmonella* serovars that cause human typhoid and other enteric fevers. Infect. Immun. **58:**2262–2275.
39. **Sola-Landa, A., J. Pizarro-Cerdá, M.-J. Grilló, E. Moreno, I. Moriyón, J.-M. Blasco, J. P. Gorvel, and I. López-Goñi.** 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol. Microbiol. **29:**125–138.
40. **Souza, V., M. Rocha, A. Valera, and L. E. Eguiarte.** 1999. Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. Appl. Environ. Microbiol. **65:**3373–3385.
41. **Sreevatsan, S., X. I. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser.** 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. USA **94:**9869–9874.
42. **Velasco, J., C. Romero, I. López-Goñi, J. Leiva, R. Díaz, and I. Moriyón.** 1998. Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. Int. J. Syst. Bacteriol. **48:**759–768.
43. **Verger, J.-M., F. Grimont, P. A. D. Grimont, and M. Grayon.** 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. **35:**292–295.
44. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.